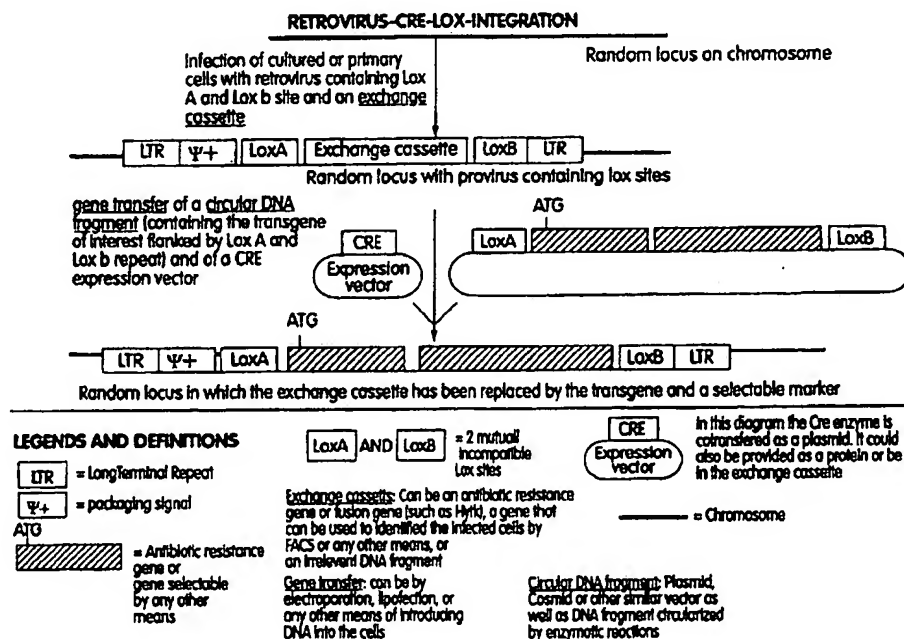




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : <b>C12N 15/86, 15/90, 15/12, C07K 14/805</b>		A1	(11) International Publication Number: <b>WO 97/47758</b>
			(43) International Publication Date: 18 December 1997 (18.12.97)
(21) International Application Number: PCT/US97/09954		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 6 June 1997 (06.06.97)		Published With international search report.	
(30) Priority Data: 08/664,084 14 June 1996 (14.06.96) US 08/743,796 5 November 1996 (05.11.96) US			
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## (54) Title: METHODS AND COMPOSITIONS FOR TRANSFORMING CELLS



## (57) Abstract

Methods and compositions for transforming cells, resulting in efficient and stable site-specific integration of transgenes, are disclosed. Transformation is achieved by introducing into a cell an acceptor vector, preferably a retroviral vector, which integrates into the genome of the cell. The acceptor vector comprises two incompatible lox sequences, L1 and L2. A donor vector is then introduced into the cell comprising a transgene flanked by the same L1 and L2 sequences. Stable gene transfer is initiated by contacting the lox L1 and L2 sequences with Cre recombinase.

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## METHODS AND COMPOSITIONS FOR TRANSFORMING CELLS

### Background of the Invention

The success of gene therapy techniques depends largely on the ability to achieve a combination of stable chromosomal integration and high-level, regulated expression of transferred genes. Regulated gene expression is most easily achieved by means of large DNA fragments containing extensive cis-acting regulatory regions. For example, gene therapy for  $\beta$ -globin disorders may require high-level, position-independent expression of extended gene and LCR sequences.

Many current techniques allow efficient transient transfection of cells *in vitro* and *in vivo* with large DNA fragments. However, subsequent chromosomal integration is very inefficient. To overcome low levels of integration, retroviral vectors which integrate very efficiently in permissive cells can be used. However, such vectors are greatly limited by constraints of size and sequence composition.

US 4,959,317 (Sauer et al.) disclose the use of Cre-Lox site-specific recombination to achieve gene transfer in eukaryotic cells. However, the system described does not provide efficient or stable integration of transferred DNA into the host genome (see e.g., (Sauer et al. (1993) *Methods in Enzymology* 225: at 898). This is largely due to the fact that excision of transferred DNA out of the genome, by way of intramolecular exchange, predominates over integration of DNA into the genome, by way of intermolecular site-specific recombination.

A site-specific DNA recombination system which allows for highly efficient and stable integration of transgene sequences into genomic DNA would be greatly beneficial.

### Brief Description of the Figures

Figure 1 is a schematic illustration of Cre/lox mediated gene transfer. Lox A and Lox B are mutually incompatible lox sites which are unable to recombine with each other in the presence of Cre recombinase.

Figure 2 is a schematic illustration of acceptor and donor vectors containing selectable marker genes and incompatible lox sequences.

Figure 3 is a schematic illustration of a Cre expression vector and a control Cre expression vector.

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**Summary of the Invention**

The present invention provides methods and compositions for causing efficient and stable site-specific DNA recombination using a recombinase/lox system, such as the Cre/lox system. In one embodiment, the method comprises contacting a recombinase (e.g., Cre) with (a) an acceptor vector comprising two incompatible lox sequences, L1 and L2, and (b) a donor vector comprising a selected DNA flanked by the L1 and L2 sequences, or sequences which are compatible with the L1 and L2 sequences, thereby causing transfer of the selected DNA from the donor vector into the acceptor vector by recombination at the compatible lox sequences. In a preferred embodiment, the acceptor vector is a retroviral vector or an adeno-associated vector.

In another embodiment, the invention provides a method of transforming a cell with a selected DNA comprising, in any order, the steps of introducing into the cell an acceptor vector which integrates into the genome of the cell, the acceptor vector comprising two incompatible lox sequences, L1 and L2, (b) introducing into the cell a donor vector comprising the selected DNA flanked by the L1 and L2 sequences, or sequences which are compatible with the L1 and L2 sequences, and (c) contacting L1 and L2 with a recombinase, such as Cre, thereby causing transfer of the selected DNA from the donor vector into the acceptor vector. The recombinase can be introduced into the cell in the form of a protein or a gene encoding the protein.

In another embodiment, the invention provides a vector selected from the group consisting of retroviral vectors and adeno-associated vectors comprising two incompatible lox sequences, L1 and L2.

The methods and compositions of the invention can be used in methods of *in vivo* and *In vitro* gene transfer (e.g., gene therapy) to cause efficient and stable integration of transgene sequences.

**Detailed Description of the Invention**

The present invention provides methods and compositions for causing efficient site-specific DNA recombination, for example, in methods of transforming cells. The advantages over current cell transformation techniques provided by the invention include highly efficient and stable integration of large DNA sequences, such as transgene sequences, into chromosomal DNA.

In one embodiment, the method of the invention comprises contacting a recombinase, such as Cre, with (a) an acceptor vector comprising two incompatible lox sequences, L1 and L2, and (b) a donor vector comprising a selected DNA flanked by the L1 and L2 sequences, or lox sequences which are compatible with the L1 and L2

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sequences, thereby causing transfer of the selected DNA from the donor vector into the acceptor vector by recombination at the compatible lox sequences.

The term "site-specific recombination," refers to DNA transfer from a donor vector to an acceptor vector.

5       The term "lox sequence" refers to a nucleotide sequence which undergoes recombination (e.g., DNA cross-over and exchange) when catalyzed by a recombinase, such as Cre or another member of the Int family of recombinases (Argos et al. (1986) *EMBO J.* 5: 433).

10       The term "recombinase" refers to any recombinase capable of catalyzing a site-specific recombination at a lox site.

      The term "incompatible lox sequences" refers to two or more lox sequences (referred to herein as L1 and L2) which differ from one another and, therefore, can not undergo recombination with one another. For example, lox sequences can be rendered incompatible if their nucleotide sequences differ by even one nucleotide, particularly in  
15       their spacer regions. In contrast, the term "compatible lox sequences" refers to two or more lox sequences which can recombine when catalyzed to do so by a recombinase.

      The term "acceptor vector" refers to any vector capable of integrating into the genome of a cell. In a preferred embodiment, the acceptor vector is of viral origin, such as a retroviral vector or adeno-associated vector. Generally, the acceptor vector contains  
20       an exchange cassette (i.e., DNA which is replaced by DNA from the donor vector) and can also, optionally, contain a selectable marker gene.

      The term "donor vector" refers to any vector (e.g., circular plasmid DNA) containing DNA which is transferred to the acceptor vector. Generally, the donor vector comprises plasmid DNA and, optionally, can contain a selectable marker gene.

25       The method of the present invention utilizes a recombinase mediated exchange reaction which takes place between identical or compatible (i.e., able to recombine with one another) lox sequences. The efficient exchange of DNA between identical or compatible lox sequences enables transfer of DNA from the donor to the acceptor vector, which each contain identical or compatible lox sites (see Figure 1). However,  
30       once transferred from donor to acceptor vector (i.e., intermolecular transfer), the transferred DNA is "locked" into place due to the incompatibility of the two lox sequences (e.g., L1 and L2) within the acceptor vector which prevent intramolecular exchange and excision of the transferred DNA. Therefore, the transferred DNA is integrated in a highly stable manner.

35       In addition to effective and stable DNA exchange reactions, the methods of the present invention take advantage of highly efficient integration vectors, such as

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retroviral vectors, adeno-associated vectors, or vectors encoding retroviral integrases, for use as acceptor vectors. The studies described herein demonstrate that such vectors are compatible for use with site-specific DNA transfer systems, such as recombinase/lox systems.

5 Overall, the site-specific recombination system of the invention provides a means for highly efficient and stable DNA transfer which can be used, for example, in methods of gene therapy. For example, the methods and compositions of the present invention can be used to achieve a 100 to 10,000 fold increase in transgene integration and expression compared to random integration which occurs in the absence of site-specific recombination.

10 Accordingly, in another embodiment, the invention provides a method of transforming a cell, such as a mammalian cell, with a selected DNA. The method comprises the steps of (a) introducing into the cell an acceptor vector comprising two incompatible lox sequences, L1 and L2, (b) introducing into the cell a donor vector  
15 comprising the selected DNA flanked by the L1 and L2 sequences, or lox sequences which are compatible with the L1 and L2 sequences, and (c) contacting L1 and L2 with a recombinase, such as Cre, to cause transfer of the selected DNA from the donor vector into the acceptor vector (by way of an exchange reaction between the compatible lox sequences). While not essential, the acceptor vector is preferably introduced into the  
20 cell prior to introduction of the donor vector, so that the acceptor vector has integrated into the host genome prior to DNA exchange with the donor vector.

The acceptor vector can be any vector capable of being taken up by cells and integrating into genomic DNA. Preferred acceptor vectors include viral vectors which transfect cells directly, such as recombinant retroviruses, adenovirus, adeno-associated  
25 virus, and herpes simplex virus-1. A prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well  
30 characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (*gag*, *pol*, *env*) has been replaced by nucleic acid encoding a mutated subunits of the mALDH of the invention rendering the retrovirus replication defective. The replication defective retrovirus is then packaged into virions  
35 which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in*

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*vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art.

- 5 Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include  $\psi$ Crip,  $\psi$ Cre,  $\psi$ 2 and  $\psi$ Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including neural cells, epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, *in vitro* and/or *in vivo* (see for example Eglitis, et al. (1985) *Science* 10 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464).

- Another preferred acceptor vector is an adenovirus-derived vector. The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. 15 (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art.

- Yet another viral vector system useful as the acceptor vector is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus 20 that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al. *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al. (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349- 25 356; Samulski et al. (1989) *J. Virol.* 63:3822-3828; and McLaughlin et al. (1989) *J. Virol.* 62:1963-1973). Vectors containing as few as 300 base pairs of AAV can be packaged and can integrate.

Other viral vector systems that may be used as the acceptor vector in the methods of the present invention include herpes virus, vaccinia virus, and several RNA viruses.

- 30 The donor vector can be any vector (e.g., circular DNA) capable of being taken up by cells, either *in vivo* or *in vitro*, and capable of carrying the desired transfer (i.e., donor) DNA sequence. Suitable donor vectors include cosmids or DNA plasmids, such as recombinant bacterial or eukaryotic plasmids. The donor vector can be introduced into the host cell either *in vivo* or *in vitro* using a variety of known methods. For *in vitro* 35 delivery, suitable methods include direct injection of the plasmid,  $\text{CaPO}_4$  precipitation, electroporation, cationic lipofection, or use of artificial viral envelopes. For *in vivo*

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delivery, suitable methods include intravenous, intraperitoneal and intramuscular injection of the vector. The vector can also be targeted for delivery to selected cells (see e.g., US 5,166,320).

To determine efficient uptake by cells, the acceptor, donor, or acceptor and donor  
5 vectors can, optionally, contain a marker gene, such as an antibiotic resistance gene or gene selectable by other means. The marker gene can be promoterless so that it will only be expressed when integrated into the acceptor vector containing a promoter to drive its expression.

The donor and acceptor vectors each contain at least two incompatible locks  
10 sequences ("L1 and L2") so that intramolecular recombination can not occur. At the same time, the locks sequences of the donor and acceptor vectors must be able to recombine intermolecularly (e.g., L1 with a compatible L1, and L2 with a compatible L2) with one another to allow DNA exchange between the donor and acceptor vectors. In order to ensure intermolecular exchange between compatible lox sequences, the lox  
15 sequences are generally oriented in the same direction.

Incompatibility between locks sequences can be achieved, for example, by way of mutating or modifying (e.g., by nucleotide addition, deletion or substitution) one of two identical lox sequences, preferably in their spacer sequences, so that the sequences differ. Testing to determine which mutations confer incompatibility can be performed  
20 using standard mutation assays which test for the ability of the mutated and non-mutated lox sequences to recombine.

In a preferred embodiment, one of the two incompatible lox sequences is the Lox P1 sequence of the Cre/lox system of bacteriophage P1 (Hoess et al. (1990) "Nucleic Acids and Molecular Biology," Vol 4, p. 99) having the sequence shown in SEQ ID NO:  
25 1. The Lox P1 sequence is a 34 base pair sequence which can be isolated from bacteriophage P1 by methods known in the art (see e.g., Hoess et al. (1982) *PNAS* 79:3398). The Lox P1 sequence consists of two 13 base pair inverted repeats separated by an eight base spacer sequence. Lox P1 sites can also be isolated from plasmids available from the ATCC (e.g., ATCC 53254 and 20773). Other suitable lox sequences  
30 include the Lox B, Lox L, and Lox R sequences isolatable from *E. coli* (Hoess et al. (1982), *supra*). Lox sequences can also be chemically synthesized using known techniques, such as those described in the Examples below.

Accordingly, the other incompatible lox sequence can be a mutated form of the LoxP1 sequence, for example, having a point mutation in the eight nucleotide spacer  
35 sequence. In one embodiment, the point mutation is substitution of A for G at position 7 of the eight base spacer sequence of the wild type Lox P1 sequence, referred to herein as



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the Lox511 sequence (SEQ ID NO: 2). Accordingly, in one embodiment, the two incompatible lox sequences of the invention have the following sequences:

SPACER

5	Lox P1	ATAACTTCGTATA ATGTAT <u>G</u> C TATACGAAGTTAT
	Lox 511	ATAACTTCGTATA ATGTATA <u>C</u> TATACGAAGTTAT

Intermolecular recombination between compatible lox sequences in the donor and acceptor vectors is catalyzed by a recombinase, such as Cre or another member of the Int family of recombinases (Argos et al. (1986) *EMBO J.* 5: 433) which have been shown to perform efficient recombination at lox sequences in both bacteria and in eukaryotic cells (Sauer et al. (1993) *Methods in Enzymology* 225: 890-900). The recombinase can be introduced into the cell along with the donor and acceptor vectors in the form of a protein or as an expressible gene encoding the protein (e.g., the Cre gene described by Sauer, B. et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:5166-5170). The recombinase or recombinase gene can be introduced or transfected into the host cell before, simultaneously, or following introduction of the donor and acceptor vectors.

In one embodiment, the recombinase gene (e.g., Cre) is contained in an expression vector which is co-transfected with the donor vector following introduction and integration of the acceptor vector into the host cell. In another embodiment, the recombinase gene is contained within either the acceptor vector or the donor vector. As with the donor vector, the recombinase gene can be introduced into the host cell either *in vivo* or *in vitro* using known techniques, such as CaPO<sub>4</sub> precipitation, electroporation, cationic lipofection, use of artificial viral envelopes, direct injection (e.g., intravenous, intraperitoneal or intramuscular). The vector can also be targeted for delivery to selected cells (see e.g., US 5,166,320).

The DNA which is transferred from the donor to the acceptor vector by way of the site-specific recombination method of the invention can be any DNA desired for stable integration into a host cell genome. For example, the gene can be any transgene useful, for example, in gene therapy or for diagnostic purposes. The gene can encode a desired therapeutic protein, such as  $\alpha$ ,  $\beta$  or  $\delta$  globin, blood coagulation factors (e.g., Factors VIII and IX) gene, cell surface receptors and other desirable proteins, for example, to correct inherited deficiencies of these proteins in an individual.

Accordingly, the methods and compositions of the invention can be used for a variety of therapeutic and diagnostic applications which require stable and efficient integration of transgene sequences into genomic DNA of cells. The methods and

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compositions can be used to transform a wide variety of eukaryotic cells (e.g., mammalian) cells and provide the advantage of high efficiency DNA transfer.

## EQUIVALENTS

5 Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. The contents of all references and published patent applications cited throughout this application are hereby incorporated by reference.

10

## EXAMPLES

### MATERIALS AND METHODS

#### 15 DNA Construction and Cell Culture

DNA vectors were made using standard techniques (Sambrook, J. et al. (1989) *Molecular Cloning: A Laboratory Manual - 2nd ed.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA). Oligonucleotides were synthesized by Research Genetics, Inc. Accuracy of DNA construction was verified by sequencing.

20 LXSXN retroviral vector (Miller, A.D. et al. (1989) *Biotechniques* 7:980-990) was provided by D. Miller (Fred Hutchinson Cancer Research Center, Seattle), Hygromycin B (Lupton, S.D. et al. (1991) *Mol. Cell. Biol.* 11:3374-3378) phosphotransferase gene by D. Housman (MIT, Cambridge), Herpes Simplex virus thymidine kinase (HSV-TK) gene (Lupton, *supra*) by M. R. Capecchi (Salt Lake City, Utah), U19 SV40T mutant

25 gene (Renfranz, P.J. et al. (1991) *Cell* 66:713-729) by R. D. McKay (MIT, Cambridge) and G. Almazan (McGill University, Montreal), Cre recombinase gene (Sauer, B. et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:5166-5170) by D. W. Ow (UC Berkeley, Albany), CD24 (Pawlink, R. et al. (1994) *Blood* 84: 2868-2877). MSCV (murine stem cell virus) retroviral vector (Hawley, P.G. et al. (1994) *Gene Therapy* 1:136-138), pBabe retroviral

30 vector (Morgenstern, J. P. et al. (1990) *Nucl. Acies Res.* 18:3587-3596) by R. Weinberg (MIT, Cambridge), pcDNA1 by Invitrogen Corp. and pOPRSVICAT by Stratagene, Inc. NIH3T3 cells were obtained from the ATCC, BOSC23 cells (Pear, W. S., et al. ((1993) *Proc. Natl. Acad. Sci., USA* 90:8392-8396) W. Pear and D. Baltimore (Rockefeller University, New York).

35 NIH3T3 cells were grown at 37°C with 5% CO<sub>2</sub>/95% air in DMEM supplemented with 10% heat inactivated calf serum (CS), 4.5 mg/ml glucose, 2 mM

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glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. For BOSC23 cells, CS was replaced by 10% heat inactivated fetal calf serum (FCS).

#### Cell Infection, Transfection and Selection

5           The packaging cell line, BOSC23, was grown as described (Pear, *supra*, Danos, O. et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464). Plasmid DNA's were prepared by the Qiagen procedure (Qiagen, Inc.) and transfected in BOSC23 cells using a calcium phosphate procedure (5prime:3prime, Inc.). Viral supernatants from producers were harvested and filtered as described (Pear, *supra*, Danos, *supra*). All  
10 infections were carried out in the presence of 8 µg/ml Polybrene (Sigma). Viral supernatants from BOSC23 were used to generate stable viral producers. Virus titers were estimated by infection and selection of NIH3T3 cells using standard calculations previously described (Pear, *supra*, Danos, *supra*). Detection of helper viruses was performed by a β-galactosidase mobilization assay as described (Pear, *supra*, Danos,  
15 *supra*). Selection was applied two days after infections. Standard concentrations (1 X) of selection agents were 320 µg/ml for Hygromycin B (Calbiochem). Packaging NIH3T3 cells were selected with 1X, MDHF with 1/2 X and BSMC with 2 X concentrations.

20

#### RETROVIRUS CRE/LOX MEDIATED GENE INTEGRATION

To study the efficiency of gene integration using the Cre/lox mediated gene transfer system described herein, the following protocol was used performed.

25           Acceptor vectors were constructed using the MSCV retroviral vector. The vectors contained in order: the left MSCV LTR (containing promoter), followed by a lox L1 sequence, followed by a hygromycin-TK fusion gene (as a selectable marker), followed by a lox L2 sequence, followed by the right MSCV LTR (see Figure 2). The retrovirus LTR was used as the promoter for the hygromycin-TK fusion gene. Similar constructs were made using other selection markers such as neomycin.

30           The L1 and L2 lox sequences of the acceptor vector had the nucleotide sequences shown below (corresponding to SEQ ID NO: 1 and SEQ ID NO: 2). L1 is the wild type LoxP1 sequence (SEQ ID NO: 1) from bacteriophage P1 (Abremski et al. (1983) *Cell* 32: 1301-1311). L2 is a mutated form of the wild type LoxP1 sequence, referred to as Lox511, having a point substitution of A for G at position 7 of the eight nucleotide  
35 spacer region (Waterhouse et al. (1993) *Nucleic Acids Res.* 21(9):2265-2266).

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SPACER

L1    ATAACTTCGTATA ATGTATGC TATACGAAGTTAT  
 L2    ATAACTTCGTATA ATGTATAC TATACGAAGTTAT

5        Following construction of the acceptor vector ("L1-hygromycin-TK-L2 construct"), BOSC23 cells (ecotropic packaging cells) were transiently transfected with acceptor vector using a calcium phosphate procedure (5prime:3prime, Inc.). Viral supernatants from producers were harvested and filtered as described (Pear, *supra*, Danos, *supra*). All infections were carried out in the presence of 8 µg/ml Polybrene  
 10        (Sigma) Viral supernatant containing high-titer ( $>10^5$  pfu/ml) retroviral vector was then used to infect host NIH 3T3 cells using the same procedures. After 48 hours in culture, the infected 3T3 cells were selected with hygromycin.

Donor vectors were constructed using pUC 19 plasmid (Yanish-Perron et al. (1985) *Gene* 33:103-119) as the backbone. The vectors contained in order: the L1 lox  
 15        sequence, followed by a promoterless neomycin gene, followed by the L2 lox sequence (see Figure 2). Similar donor vectors were made using hygromycin-TK, CD24, and B-globin genes in place of the neomycin gene. Control donor vectors were constructed using a neomycin gene with the PGK (phosphoglycerol kinase) promoter, PGK-neomycin.

20        Various concentrations of donor vector containing neomycin gene were co-electroporated, along with an expression vector containing the Cre recombinase gene, into the infected 3T3 cells. The concentrations of donor vector ranged from 10 µg to 200 µg. After 48 hours in culture, transformed cells were selected with neomycin. Concentrations 100 µg or more of donor vector resulted in a 10-30% integration  
 25        efficiency (as measured by transfer of neomycin gene for hygromycin gene).

Different ratios of donor vector and Cre expression vector, ranging from 20:1 to 1:1 were co-electroporated into the infected 3T3 cells. All ratios resulted in the transfer of the neomycin for the hygromycin. However, a ratio of 3:1 (donor:Cre) resulted in the highest integration efficiency.

30        The following table provides the results of neomycin gene integration using various donor and Cre expression vectors (see Figure 3) at a concentration of 100 µg of donor vector (DNA) at a ratio of 3 parts donor vector to 1 part Cre expression vector. Experiments E#1-4 were performed as negative controls. E#5 was the positive control.

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	Cells Electroporated		Constructs Used	# Colonies (out of $10^7$ cells)
5	E#1	3T3	lox 1-PGKNeo-lox 2 & control Cre expression vector	530
10	E#2	3T3	lox 1-Neo-lox 2 & control Cre expression vector	10
	E#3	3T3	lox 1-Neo-lox 2 & Cre expression vector	2
15	E#4	3T3 containing lox A-hygro-TK-lox B	lox 1-Neo-lox 2 & control Cre expression vector	21
	E#5	same	lox 1-Neo-lox 2 & Cre expression vector	confluent ( $>10^5$ )
20	<p><b>E#1</b> used the control donor vector (see Figure 2), lox 1-PGKNeo-lox 2 (containing the neomycin gene and a promoter) along with a control Cre expression vector (see Figure 3) (in which the sequence encoding Cre had been deleted and replaced by a gene encoding CAT). Host cells did not contain integrated acceptor vector. Therefore, E#1</p>			
25	<p>demonstrated the amount of neomycin resistance conferred by random integration of the L1-PGKNeo-L2 vector capable of expressing the neomycin gene. As expected, the conferred neomycin resistance was in the range of efficiency of integration obtained by electroporation (e.g., about 0.1% efficiency).</p>			
30	<p><b>E#2</b> used donor vector (promoterless) with a control Cre expression vector. Host cells did not contain integrated acceptor vector. Therefore, E#2 demonstrated the resistance conferred in the absence of acceptor vector or Cre recombinase (i.e., in the absence of efficient recombination and gene transfer). As expected, this was very low.</p>			
35	<p><b>E#3</b> used donor vector (promoterless) with a functional Cre expression vector. Host cells did not contain integrated acceptor vector. Therefore, E#3 demonstrated the resistance conferred in the absence of acceptor vector (i.e., in the absence of efficient recombination and gene transfer), but in the presence of Cre recombinase. As expected, this was very low.</p>			

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- 12 -

E#4 used donor vector (promoterless) with a control Cre expression vector (no Cre expression). Host cells contained integrated acceptor vector (L1-hygro-TK-L2). Therefore, E#4 demonstrated the gene transfer efficiency from donor vector to acceptor vector in the absence of Cre). As expected, this was very low.

5

E#5 used donor vector (promoterless) with a functional Cre expression vector. Host cells contained integrated acceptor vector (L1-hygro-TK-L2). Therefore, E#5 demonstrated the gene transfer efficiency from donor vector to acceptor vector in the presence of Cre. As shown in the table above, the host cells became confluent, demonstrating a greater than 1000 fold increase in gene transfer efficiency and stability.

10

**Conclusion:**

Overall, these results demonstrate that the retroviral Cre/lox mediated gene transfer system of the present invention can be used for highly efficient and stable integration of transgenes into chromosomal DNA of mammalian cells.

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- 13 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

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(ii) TITLE OF INVENTION: METHODS AND COMPOSITIONS FOR  
TRANSFORMING CELLS

20

## (iii) NUMBER OF SEQUENCES: 2

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30

## (v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- 35 (D) SOFTWARE: ASCII text

35

## (vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: PCT/US97/
- (B) FILING DATE: 06 JUNE 1997
- 40 (C) CLASSIFICATION:

40

## (vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/743,796
- (B) FILING DATE: 05 NOVEMBER 1996
- 45 (C) APPLICATION NUMBER: US 08/664,084
- (D) FILING DATE: 14 JUNE 1996

45

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- (B) REGISTRATION NUMBER: 38,872
- (C) REFERENCE/DOCKET NUMBER: MTE-198PC

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- 14 -

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(2) INFORMATION FOR SEQ ID NO:1:

5

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 34 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: DNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATAACTTCGT ATAATGTATG CTATACGAAG TTAT

34

20

(2) INFORMATION FOR SEQ ID NO:2:

25

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 34 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

35 ATAACTTCGT ATAATGTATA CTATACGAAG TTAT

34



- 15 -

What is claimed is:

1. A method of causing a site-specific recombination comprising contacting a recombinase with (a) an acceptor vector comprising two incompatible lox sequences, L1 and L2, and (b) a donor vector comprising a selected DNA flanked by the L1 and L2 sequences, or sequences which are compatible with the L1 and L2 sequences, thereby causing transfer of the selected DNA from the donor vector into the acceptor vector by recombination at the compatible lox sequences.
2. The method of claim 1 wherein the acceptor vector is selected from the group consisting of retroviral vectors and adeno-associated vectors.
3. The method of claim 1 wherein L1 and L2 differ by one or more point mutations in their spacer sequences, rendering them incompatible.
4. The method of claim 1 wherein L1 and L2 comprise the LoxP1 and Lox511 nucleotide sequences (SEQ ID NOS:1 and 2) and the recombinase is Cre.
5. The method of claim 1 wherein acceptor vector comprises a retroviral vector.
6. A method of transforming a cell with a selected DNA comprising, in any order, the steps of:
  - (a) introducing into the cell an acceptor vector which integrates into the genome of the cell, the acceptor vector comprising two incompatible lox sequences, L1 and L2;
  - (b) introducing into the cell a donor vector comprising the selected DNA flanked by the L1 and L2 sequences, or sequences which are compatible with the L1 and L2 sequences; and
  - (c) contacting L1 and L2 with Cre, thereby causing transfer of the selected DNA from the donor vector into the acceptor vector.
7. The method of claim 6 wherein the acceptor vector comprises an adeno-associated viral vector.

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8. The method of claim 6 wherein L1 and L2 differ by at least one nucleotide addition, deletion or substitution in their spacer sequences, rendering them incompatible.

5 9. The method of claim 6 wherein either L1 or L2 comprises the LoxP1 nucleotide sequence (SEQ ID NO:1).

10 10. The method of claim 6 wherein either L1 or L2 comprises the Lox511 nucleotide sequence (SEQ ID NO:2).

11 11. The method of claim 6 wherein L1 and L2 comprise the LoxP1 and Lox511 nucleotide sequences (SEQ ID NOS:1 and 2).

12 12. The method of claim 6 wherein L1 and L2 have the same orientation.

15 13. The method of claim 6 further comprising the step of introducing into the cell a gene encoding Cre recombinase.

20 14. The method of claim 13 wherein the gene encoding Cre recombinase is contained in an expression vector.

15 15. The method of claim 13 wherein the gene encoding Cre recombinase is contained in either the acceptor vector or the donor vector.

25 16. The method of claim 6 wherein the donor vector is introduced into the cell by electroporation.

30 17. The method of claim 13 wherein both the donor vector and the gene encoding Cre recombinase are introduced into the cell by electroporation.

18. The method of claim 6 wherein the selected DNA is a transgene encoding a therapeutic protein.

35 19. The method of claim 18 wherein the protein is  $\beta$ -globin.

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20. The method of claim 6 wherein the donor vector, acceptor vector, or both the donor and acceptor vectors further comprise a selectable marker gene.

21. The method of claim 6 wherein the cell is a mammalian cell.

5

22. A vector selected from the group consisting of retroviral vectors and adeno-associated vectors comprising two incompatible lox sequences, L1 and L2.

23. The vector of claim 22 wherein L1 and L2 comprise the LoxP1 and Lox511 nucleotide sequences (SEQ ID NOS:1 and 2).

10

24. The vector of claim 22 wherein the vector further comprises a selectable marker gene.

15

25. A Cre/lox mediated gene transfer system comprising:

(a) an acceptor vector which can integrate into the genome of a host cell, the acceptor vector comprising two incompatible lox sequences, L1 and L2;

(b) a donor vector comprising a transgene flanked by the L1 and L2 sequences or sequences which are also compatible with the L1 and L2 sequences; and

20

(c) a recombinase which can catalyze recombination between the compatible lox sequences.

25

26. The system of claim 25 wherein the acceptor vector is selected from the group consisting of retroviral vectors and adeno-associated vectors.

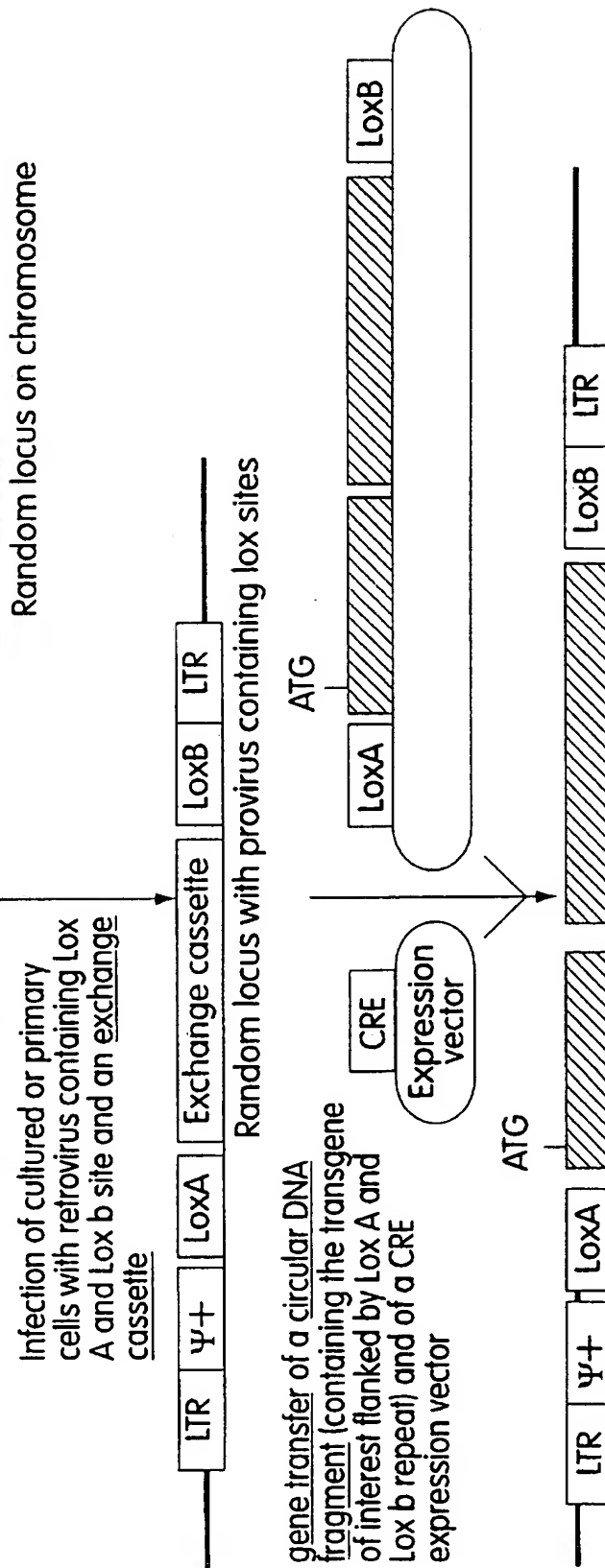
27. The system of claim 25 wherein L1 and L2 differ by one or more point mutations in their spacer sequences, rendering them incompatible.

30

28. The system of claim 25 wherein L1 and L2 comprise the LoxP1 and Lox511 nucleotide sequences (SEQ ID NOS:1 and 2).

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# RETROVIRUS-CRE-LOX-INTEGRATION



Random locus in which the exchange cassette has been replaced by the transgene and a selectable marker

## LEGENDS AND DEFINITIONS

**LTR** = Long Terminal Repeat  
**ψ+** = packaging signal  
**ATG** = Antibiotic resistance gene or gene selectable by any other means

**LoxA AND LoxB** = 2 mutually incompatible Lox sites

**Exchange cassettes:** Can be an antibiotic resistance gene or fusion gene (such as Hyg<sup>r</sup>), a gene that can be used to identify the infected cells by FACS or any other means, or an irrelevant DNA fragment

**Gene transfer:** can be by electroporation, lipofection, or any other means of introducing DNA into the cells

In this diagram the Cre enzyme is cotransferred as a plasmid. It could also be provided as a protein or be in the exchange cassette

**CRE** Expression vector

— = Chromosome

**Circular DNA fragment:** Plasmid, Cosmid or other similar vector as well as DNA fragment circularized by enzymatic reactions

Fig. 1

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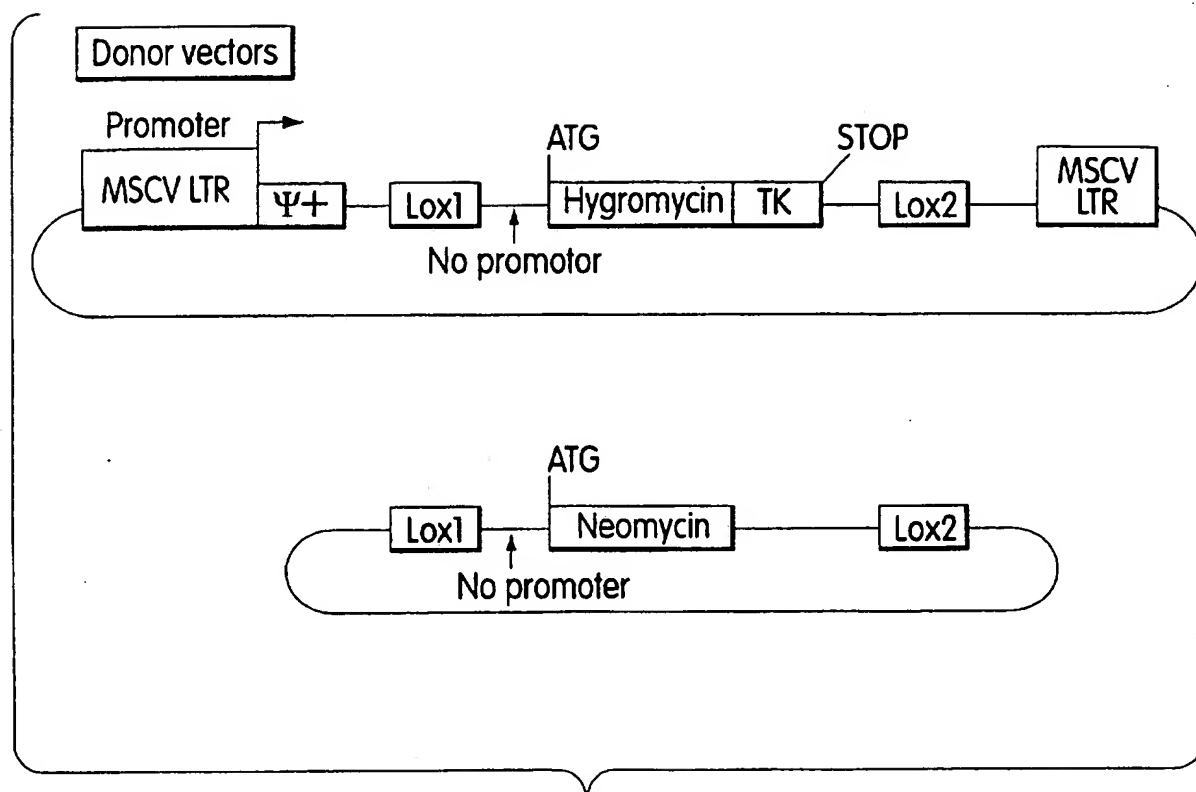


Fig. 2

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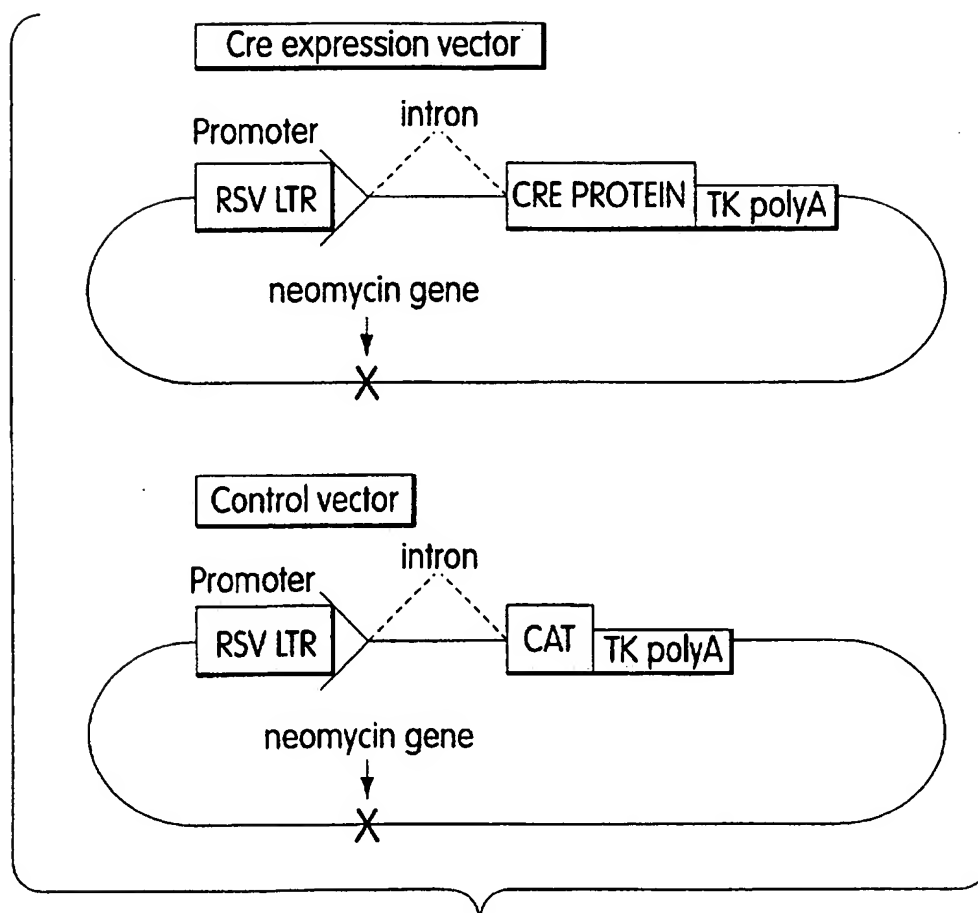


Fig. 3

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 97/09954

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/86 C12N15/90 C12N15/12 C07K14/805

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NUCLEIC ACIDS RESEARCH, vol. 21, no. 9, 11 May 1993, page 2265/2266 XP000575849 WATERHOUSE P ET AL: "COMBINATORIAL INFECTION AND IN VIVO RECOMBINATION: A STRATEGY FOR MAKING LARGE PHAGE ANTIBODY REPERTOIRES" cited in the application see the whole document ---	1,4
A	SOMATIC CELL AND MOLECULAR GENETICS, vol. 21, no. 6, November 1995, pages 429-441, XP000617918 WANG P ET AL: "HIGH FREQUENCY RECOMBINATION BETWEEN LOXP SITES IN HUMAN CHROMOSOMES MEDIATED BY AN ADENOVIRUS VECTOR EXPRESSING CRE RECOMBINASE" see the whole document --- -/-	1-28

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
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- \*O\* document referring to an oral disclosure, use, exhibition or other means
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\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*G\* document member of the same patent family

Date of the actual completion of the international search

5 September 1997

Date of mailing of the international search report

16. 09. 97

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## INTERNATIONAL SEARCH REPORT

Inter nal Application No

PC1/US 97/09954

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF VIROLOGY, vol. 70, no. 3, March 1996, pages 1792-1798, XP000616291 CHOULIKA A ET AL: "TRANSFER OF SINGLE GENE-CONTAINING LONG TERMINAL REPEATS INTO THE GENOME OF MAMMALIAN CELLS BY A RETROVIRAL VECTOR CARRYING THE CRE GENE AND THE LOXP SITE" see the whole document ---	1-28
A	EP 0 300 422 A (DU PONT) 25 January 1989 see the whole document ---	1-28
A	WO 93 09239 A (RES CORP TECHNOLOGIES INC) 13 May 1993 see the whole document ---	1-28
A	US 4 959 317 A (SAUER BRIAN L) 25 September 1990 cited in the application see the whole document ---	1-28
P,A	WO 97 06271 A (PASTEUR INSTITUT ;CHOULIKA ANDRE (FR); NICOLAS JEAN FRANCOIS (FR)) 20 February 1997 see the whole document ---	1-28
P,X	THIRTY-EIGHTH ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY, ORLANDO, FLORIDA, USA, DECEMBER 6-10, 1996. BLOOD 88 (10 SUPPL. 1 PART 1-2). 1996. 688A. ISSN: 0006-4971, XP002039898 LEBOULCH P ET AL: "Retrovirus and Cre -Lox-mediated integration (RCLI): A two-step gene transfer procedure for efficient chromosomal integration of large DNA fragments." abstract no. 2739 see abstract ---	1-28
P,X	THIRTY-EIGHTH ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY, ORLANDO, FLORIDA, USA, DECEMBER 6-10, 1996. BLOOD 88 (10 SUPPL. 1 PART 1-2). 1996. 190A. ISSN: 0006-4971, XP002039899 BOUHASSIRA E E ET AL: "Recombinase-mediated-cassette-exchange (RMCE): A novel technique for integration of single copy of transgenes at pre-determined chromosomal sites. Application to the study of the human beta-globin LCR." abstract no. 749 see abstract -----	1-28



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/09954

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0300422 A	25-01-89	AU 610608 B	23-05-91
		AU 1920188 A	27-01-89
		DE 3876327 A	14-01-93
		JP 1112986 A	01-05-89
-----			
WO 9309239 A	13-05-93	US 5252479 A	12-10-93
		AU 657829 B	23-03-95
		AU 3134093 A	07-06-93
		CA 2098483 A	09-05-93
		EP 0566732 A	27-10-93
		JP 6504680 T	02-06-94
		PT 101040 A	31-05-94
-----			
US 4959317 A	25-09-90	CA 1293460 A	24-12-91
		DE 3687734 A	25-03-93
		EP 0220009 A	29-04-87
		ES 2053438 T	01-08-94
		IE 60421 B	13-07-94
		JP 62175184 A	31-07-87
-----			
WO 9706271 A	20-02-97	FR 2737731 A	14-02-97
		AU 6703396 A	05-03-97
-----			